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APPLICATION FOR UNITED STATES PATENT

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William T. Chance

Title: COMPOUNDS FOR CONTROL OF APPETITE, BLOOD
PRESSURE, CARDIOVASCULAR RESPONSE,
LIBIDO, AND CIRCADIAN RHYTHM

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SPECIFICATION

**COMPOUNDS FOR CONTROL OF APPETITE, BLOOD PRESSURE,
CARDIOVASCULAR RESPONSE, LIBIDO, AND CIRCADIAN RHYTHM**

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**Ambikaipakan Balasubramaniam
William T. Chance**

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~~This application is a continuation-in-part of U.S. Application Serial No. 09/449,914, filed on December 2, 1999, which is a divisional of U.S. Patent No. 6,013,633, issued January 11, 2000.~~

Background of the Invention

This invention relates generally to dipeptides and tripeptides and to methods for pharmaceutical treatment of mammals using analogs of such dipeptides and tripeptides. More specifically, the invention relates to tripeptides and their analogs, to pharmaceutical compositions containing such dipeptides and tripeptides and to methods of treatment of mammals using such dipeptides and tripeptides. In addition, the invention relates to methods of treatment of mammals using such dipeptides and tripeptides for control of appetite, blood pressure, cardiovascular response, libido, and circadian rhythm.

1. Appetite and obesity

Obesity is a major disorder affecting as much as one third of the North American population. Several studies have shown that such individuals are at increased risk in developing cardiovascular disease (hypertension and hypercholesterolemia), diabetes and several types of cancer. The effective treatment of obesity, however, remains a largely unachieved goal. Existing pharmacotherapeutic approaches to weight loss involve the use of amphetamine-based agents such as amphetamine, diethylpropion, mazindol and fenfluramine which act directly on the CNS to lower food intake by modulating dopaminergic, adrenergic and/or serotonergic mechanisms. Although weight loss can be achieved with such agents, their use is restricted due to CNS side-effects, potential addiction liability and the production of tolerance to their actions, with chronic administration leading to potential depression, vestibular disturbances, hallucinations and addiction, as well as interference with the actions other drugs such as MAO inhibitors and antihypertensives. There is also a subpopulation of obese patients that is refractory to present anorectic drug treatments. The medical need is high for an effective anorectic agent which overcomes the above disadvantages of existing therapies. Of particular need are agents which act by alternative mechanisms to modulate food intake and/or metabolism.

2. Neuropeptide Y ("NPY")

Throughout this application, various publications are referenced. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

- Neuropeptides are small peptides originating from large precursor proteins synthesized by peptidergic neurons and endocrine/paracrine cells. They hold promise for treatment of neurological, psychiatric, and endocrine disorders (De Wied, D. In: *Neuropeptides: Basics and Perspectives* (Elsevier, Amsterdam-New York-Oxford), 1990.). Often the precursors contain multiple biologically active peptides.
- 5 There is great diversity of neuropeptides in the brain caused by alternative splicing of primary gene transcripts and differential precursor processing. The neuropeptide receptors serve to discriminate between ligands and to activate the appropriate signals. Thus, it is expected that the receptors for neuropeptides consist of a large number of
- 10 members.

- Neuropeptide Y (NPY), a 36-amino acid peptide, is the most abundant neuropeptide to be identified in mammalian brain. NPY is an important regulator in both the central and peripheral nervous systems (Heilig, M. and E. Widerlov. Neuropeptide Y: an overview of central distribution, functional aspects, and possible
- 15 involvement of neuropsychiatric illnesses. *Acta Psychiatr. Scand.* 82:95-114 (1990).) and influences a diverse range of physiological parameters, including effects on psychomotor activity, food intake, central endocrine secretion, and vasoactivity in the cardiovascular system. High concentrations of NPY are found in the sympathetic nerves supplying the coronary, cerebral, and renal vasculature and has contributed
- 20 vasoconstriction. NPY binding sites have been identified in a variety of tissues, including spleen (Lundberg, J. M., A. Hemsén, O. Larsson, A. Rudehill, A. Saria, and B. Fredholm. Neuropeptide Y receptor in pig spleen: binding characteristics, reduction of cyclic AMP formation and calcium antagonist inhibition of

- vasoconstriction. *Eur. J. Pharmacol.* 145:21-29 (1988)), intestinal membranes, brain (Hinson, J., C. Rauh, and J. Coupet. Neuropeptide Y stimulates inositol phospholipid hydrolysis in rat brain microprisms. *Brain RESPONSE*. 446:379-382 (1988)), aortic smooth muscle (Mihara, S., Y. Shigeri, and M. Fujimoto. Neuropeptide Y-induced intracellular Ca^{2+} increase in vascular smooth muscle cells. *FEBS Lett.* 259: 79-82 (1989)), kidney, testis, and placenta (Dumont, Y., J.C. Martel, A. Fournier, S. St.-Pierre, and R. Quiron. Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. *Prog. Neurobiol.* 38:125-167 (1992)). In addition, binding sites have been reported in a number of rat and human cell lines (e.g. Y1 in SK-N-MC, MC-IXC, CHP-212, and PC12 cells; Y2 in SK-N-BE(2), CHP-234, and SMS-MSN)(Grundemar, L., S. P. Sheikh, and C. Wahlestedt, In: *The Biology of Neuropeptide Y and Related Peptides*. (Humana Press, Inc., Totawa, New Jersey), (1992)).

- NPY forms a family (called the pancreatic polypeptide family) together with pancreatic polypeptide (PP) and peptide YOSHIOKA (PYY) which all consist of 36 amino acids and have a common tertiary structure, the so-called PP-fold (Glover, I. D., D. J. Barlow, J. E. Pitts, S. P. Wood, I. J. Tickle, T. L. Blundell, K. Tatamoto, J. R. Kimmel, A. Wollmer, W. Strassburger, and Y.-S. Zhang. Conformational studies of the pancreatic polypeptide hormone family. *Eur. J. Biochem.* 142:379-385 (1985)). Specific features of this family include a polyproline helix in residues 1 through 8, beta-turn in residues 9 through 14, an alpha-helix in residues 15 through 30, an outward-projecting C-terminus in residues 30 through 36, and a carboxy terminal amide which appears to be critical for biological activity (Schwartz, T. W., J.

- Fuhlendorff, L. L. Kjems, M. S. Kristensen, M. Vervelde, M. O'Hare, J. L. Krstenansky, and B. Bjornholm. Signal epitopes in the three-dimensional structure of neuropeptide Y. *Ann. N.Y. Acad. Sci.* 611:35-47 (1990)). The C-terminal amidated residue of these peptides is essential for biological activity (Wahlestedt *et al.*, 1986).
- 5 Studies with peptide fragments of NPY have indicated that multiple NPY receptor subtypes exist (Wahlestedt, C., N. Yanaihara, and R. Hakanson. Evidence for different pre- and postjunctional receptors for neuropeptide Y and related peptides. *Regul. Pept.* 13:307-318 (1986)). Three major NPY receptor subtypes (Y1, Y2 and Y3) have been defined by pharmacological criteria, with a fourth "atypical" Y1
 - 10 receptor that has been proposed to regulate feeding behavior. One of the key pharmacological features which distinguish Y1 and Y2 is the fact that the Y1 receptor (and not the Y2 receptor) responds to an analog of NPY modified at residues 31 and 34 ([Leu31,Pro34]NPY), whereas the Y2 receptor (and not the Y1 receptor) has high affinity for the NPY peptide carboxyl-terminal fragment NPY-(13-36)(Fuhlendorff, J.,
 - 15 U. Gether, L. Aakerlund, N. Langeland-Johansen, H. Thøgersen, S. G. Melberg, U. B. Olsen, O. Thastrup, and T. W. Schwartz. [Leu31,Pro34]Neuropeptide Y: A specific Y1 receptor agonist. *Proc. Natl. Acad. Sci. USA* 87:182-186 (1990)).

- Experimental and clinical observations have supported the concept that neuropeptides play central roles in neurotransmission as well as the regulation of
- 20 secretory functions of adenohipophysial, pancreatic, adrenalcortical and gut cells. Among the thirty or so neuropeptides that have been implicated in neuronal function in the mammalian central nervous system, several have also been suggested to function as neurotransmitters or neuromodulators primarily in afferent neurons.

An additional action of NPY is to decrease cardiac contractility (inotropy). This is an extremely important action of NPY, because it is known that, under many circumstances in which inotropy is decreased, diseases of life-threatening importance, *e.g.* congestive heart failure and cardiogenic shock, are associated with probable increased release of NPY into the blood. Prevention of NPY release, using a presynaptic NPY agonist, or NPY's action, using a postsynaptic NPY antagonist, may be beneficial in these disease states.

NPY has also been reported to produce coronary artery vasoconstriction and thereby may decrease myocardial blood flow resulting in myocardial ischemia. Such a circumstance can result in angina pectoris or, under more severe circumstances, may result in myocardial infarction and death. In recent years, several classes of drugs have proven effective in dilating coronary arteries to prevent such events. The use of analogs of NPY are expected to prove useful in treatment of such problems.

U.S. Pat. No. 4,297,346, Rips, discloses therapeutic agents referred to as 'pseudopeptides' being formed from at least one peptide radical connected by a peptide bond to a therapeutically active molecule or derivative of a therapeutically active molecule. The therapeutic agents of the invention may be in the form of derivatives such as salts, esters and amides. The basis of action of the agents of the invention is the ability of the agents of the invention to cross bodily biological barriers because of the basically peptide structure of the agents. The invention also includes the preparation of the agents of the invention.

dis 21
U.S. Pat. No. 5,328,899, Douljik *et al.*, issued Jul. 12, 1994, discloses

NPY peptide analogs. Human Neuropeptide Y (NPY) has the formula: H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH₂. Porcine
5 and rat NPY have the same sequence except for Leu instead of Met in the 17-position. Porcine PYY is homologous having 11 different residues. NPY analogs and N-terminally-shortened fragments, *e.g.* NPY(18-36), which contain one or more specific D-isomer substitutions for the naturally occurring residues (as well as pharmaceutically acceptable salts thereof), dispersed in a pharmaceutically acceptable
10 liquid or solid carrier, can be administered to mammals, including humans, to substantially lower blood pressure over an extended period of time or to counteract ~~hypertension~~.

U.S. Pat. No. 4,839,343, Waeber *et al.*, issued Jun. 13, 1989, discloses a preparation containing hexatriacontapeptides suitable for intravenous administration
15 to human or other animal subjects which are effective in treating life-threatening hypotension as encountered in bacteremic, anaphylactic or cardiogenic shock.

Several references have disclosed CCK agonists or analogs of CCK-8. For example, U.S. Pat. No. 4,490,364, issued Dec. 25, 1984 to Rivier, discloses heptapeptide, octapeptide and nonapeptide analogs of CCK-8 as CCK agonists for
20 stimulating gallbladder contractions, arresting the secretion of gastric acid and treating convulsions. J. D. Rosamond in European Patent Application EP381,340, published Aug. 8, 1990, and in European Patent Application EP268,297, published May 25,

1988, discloses hepta- and octapeptides with sulfate ester groups which are useful for treating obesity.

U.S. Pat. No. 5,270,302, Shiosaki *et al.*, issued Dec. 14, 1993,

- discloses derivatives of tetrapeptides as CCK agonists which are selective and potent
- 5 Type-A CCK receptor agonists useful in the treatment of gastrointestinal disorders (including gallbladder disorders), central nervous system disorders, insulin-related disorders and pain, as well as in appetite regulation.

None of these references individually or collectively teach or suggest the present invention.

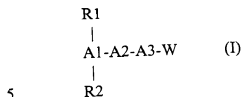
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Summary of the Invention

- This invention relates generally to dipeptides and tripeptides and to methods for pharmaceutical treatment of mammals using analogs of such dipeptides and tripeptides. More specifically, the invention relates to tripeptides and their
- 15 analogs, to pharmaceutical compositions containing such dipeptides and tripeptides and to methods of treatment of mammals using such dipeptides and tripeptides. In addition, the invention relates to methods of treatment of mammals using such dipeptides and tripeptides for control of appetite, blood pressure, cardiovascular response, libido, and circadian rhythm.

20

The present invention is directed to neuropeptide Y ("NPY") receptor antagonists and agonists. In one aspect, the present invention features compounds having the formula:



wherein:

- each R1 and R2, independently, is H, C1-C12 alkyl (*e.g.*, methyl), C6-C18 aryl (*e.g.*, phenyl), C1-C18 acyl (*e.g.*, formyl, acetyl, and myristoyl), C7-C18 aralkyl (*e.g.*, benzyl), C7-C18 alkaryl (*e.g.*, p-methylphenyl) or a dihydrotrigonellinate group;

- ~~A1 is a D or L-amino acid selected from the group consisting of Cys, Leu, Dap, Trp, Gln, a tethered amino acid with an indole ring (*e.g.*, N-Me-Trp), Phe, Hyp, any Trp derivative (*e.g.*, 2 chlorotryptophan, or Tcc); CaMe-Trp, CaMe-Gln, Des-amino-Trp, Pyr, Bth, Nal, Tcc, Asn, Nva, Abu, Tyr, Tic-OH, Phe, Tip, and Dip;~~

- A2 is a D or L-amino acid selected from the group consisting of Cys, Trp, Arg, N-Me-Arg, CaMe-Arg, Orn, Cit, hArg(R)2 [where R is selected from the group consisting of hydrogen, alkyl, aryl, aralkyl, or alkylaryl], Lys-ε-NH-R [where R is selected from the group consisting of hydrogen, alkyl, aryl, aralkyl, or alkylaryl];

- A3 is a D or L-amino acid selected from the group consisting of Glu, N-Me-Tyr, CaMe-Tyr, Tic-OH, Tic, Dip, Trp, Phe, des-carboxylic-Tyr (tyramine), and Tyr-(R) [where R is hydrogen or a lipophilic group, *e.g.*, myristoyl, cholesteryl, tBu, *etc.*];

W is -OH, -N-R3R4, or OR5 (where R3, R4, and R5, independently, is H, C1-C12 alkyl (e.g., methyl), C6-C18 aryl (e.g., phenyl), C1-C12 acyl (e.g., formyl, acetyl, and myristoyl), C7-C18 aralkyl (e.g., benzyl), or C7-C18 alkaryl (e.g., p-methylphenyl); or a pharmaceutically acceptable salt thereof; and

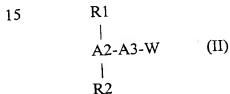
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each bond between two amino acids or amino acid derivatives, represented by a dash ("—"), can be either a peptide bond or a pseudopeptide bond or a pharmaceutically acceptable salt thereof.

10 ~~Preferred compounds of formula (I) include those in which A1 is Try or a pharmaceutically acceptable salt thereof.~~

In another aspect, the invention features a compound having the

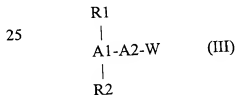
formula:



20

In another aspect, the invention features a compound having the

formula:



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In another aspect, the invention features a compound having the

formula:

Cyclo-[-A1-A2-A3-],

(IV)

Cyclo-[-A1-A2-A3-A1-A2-A3-], or

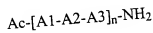
(V)

Cyclo-[-A1-A2-A3-A3-A2-A1-].

(VI)

In another aspect, the invention features a compound having the

5 formula:



(VII)

(n = 1, 2, 3) (A Tandem Peptide)

In another aspect, the invention features dimers of compounds having

the formula (I-III). Dimers may be prepared by dimerizing compounds of formula (I-
10 III) with dicarboxylic acids (e.g., succinic acid), cystine, or diaminodicarboxylic acid
(e.g., 2,6-diaminopimelic acid).

In another aspect, the invention features a compound having the
formula of (I-VII) conjugated to carriers.

In another aspect, the invention features a method of controlling the
15 blood pressure of a subject experiencing hypertension.

In another aspect, the invention features a method of controlling a NPY
physiological response in the cardiovascular system, including blood pressure, of a
subject.

In other preferred embodiments, a compound of formula (selected from
20 I thru VII) may have one or more pseudopeptide bonds. The abbreviation psi ("ψ") is
the designation for a pseudopeptide bond (e.g., ("ψ")("CH₂NH")) indicates that the
amide ("C(O)NH-") bond of a peptide has been replaced by the reduced form ("CH₂NH-").

In other preferred embodiments, a therapeutically effective amount of a compound of formula (selected from I thru VII) and a pharmaceutically acceptable carrier substance together form a therapeutic composition capable of suppressing an NPY mediated physiological response.

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Brief Description of the Figures

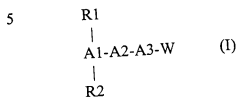
Figure 1. A graph representing the feeding patterns of animals treated with the tripeptides by intrahypothalamic cannulae. The compounds tested include
10 control (artificial cerebral spinal fluid, CSF), T-83 (Ac-Tcc-Arg-Tyr-NH₂), T-84 (N-a-indole-3-acetyl-Arg-Tyr-NH₂), and T-91 (Ac-Trp-Arg-Tic(OH)-NH₂).

Figure 2. A graph representing the feeding patterns of animals treated with the tripeptides by intrahypothalamic cannulae. The compounds tested include
15 control (artificial cerebral spinal fluid, CSF), T-45 (Bis(34/34') {[Cys34]NPY(34-36)}), WX-66 (Ac-D-Trp-Arg-Tyr-NH₂), and WX-126 (Ac-Gln-Arg-Tyr-NH₂).

Figure 3. A graph representing the feeding patterns of animals treated with the tripeptides by intrahypothalamic cannulae. The compounds tested include
20 control (artificial cerebral spinal fluid, CSF) and T-34 (N-Ac-Trp-Arg-Tyr-NH₂).

Detailed Description of the Invention

The present invention is directed to neuropeptide Y ("NPY") receptor antagonists and agonists. In one aspect, the present invention features compounds having the formula:



10 wherein:

each R1 and R2, independently, is H, C1-C12 alkyl (*e.g.*, methyl), C6-C18 aryl (*e.g.*, phenyl), C1-C18 acyl (*e.g.*, formyl, acetyl, and myristoyl), C7-C18 alkaryl (*e.g.*, benzyl), C7-C18 alkaryl (*e.g.*, p-methylphenyl) or a dihydrotrigonellinate group;

15 A1 is a D or L-amino acid selected from the group consisting of Cys, Leu, Dap, Trp, Gln, a tethered amino acid with an indole ring (*e.g.*, N-Me-Trp), Phe, Hyp, any Trp derivative (*e.g.*, 2 chlorotryptophan, or Tcc); CaMe-Trp, CaMe-Gln, Des-amino-Trp, Pyr, Bth, Nal, Tcc, Asn, Nva, Abu, Tyr, Tic-OH, Phe, Tip, and Dip;

20 A2 is a D or L-amino acid selected from the group consisting of Cys, Trp, Arg, N-Me-Arg, CaMe-Arg, Orn, Cit, hArg(R)₂ [where R is selected from the group consisting of hydrogen, alkyl, aryl, aralkyl, or alkylaryl], Lys-ε-NH-R [where R

is selected from the group consisting of hydrogen, alkyl, aryl, aralkyl, or alkylaryl];

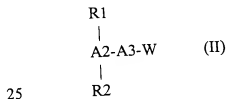
A3 is a D or L-amino acid selected from the group consisting of Glu, N-Me-Tyr,
CaMe-Tyr, Tic-OH, Tic, Dip, Trp, Phe, des-carboxylic-Tyr (tyramine), and Tyr-
5 (R) [where R is hydrogen or a lipophilic group, *e.g.*, myristoyl,olesteryl, t.Bu,
etc.];

W is -OH, -N-R3R4, or OR5 (where R3, R4, and R5, independently, is H, C1-C12
alkyl (*e.g.*, methyl), C6-C18 aryl (*e.g.*, phenyl), C1-C12 acyl (*e.g.*, formyl,
10 acetyl, and myristoyl), C7-C18 aralkyl (*e.g.*, benzyl), or C7-C18 alkaryl (*e.g.*, p-
methylphenyl); or a pharmaceutically acceptable salt thereof; and

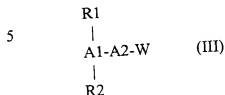
each bond between two amino acids or amino acid derivatives, represented by a
dash ("-"), can be either a peptide bond or a pseudopeptide bond or a
15 pharmaceutically acceptable salt thereof.

Preferred compounds of formula (I) include those in which A1 is Try or a
pharmaceutically acceptable salt thereof.

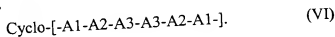
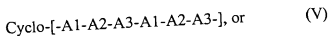
In another aspect, the invention features a compound having the
20 formula:



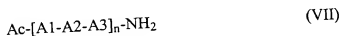
In another aspect, the invention features a compound having the formula:



10 In another aspect, the invention features a compound having the formula:



15 In another aspect, the invention features a compound having the formula:



(n = 1, 2, 3) (A Tandem Peptide)

20 In another aspect, the invention features dimers of compounds having the formula I-III) prepared by dimerizing the compound with dicarboxylic acids (*e.g.*, succinic acid), cystine, or diaminodicarboxylic acid (*e.g.*, 2,6-diaminopimelic acid).

In another aspect, the invention features a compound having the formula of (I) could be conjugated to carriers, *e.g.*, cationized albumin
25 (Endocrinology 126:977-984 (1990); J. Pharmacol Exp. Therao. 268:791-796 (1994), incorporated herein by reference in their entirety) or polylysine, *e.g.*, MAP.

In another aspect, the invention features a method of controlling the blood pressure of a subject experiencing hypertension which comprises administering to said subject the compound of formula (I-VII).

In another aspect, the invention features a method of controlling a NPY
5 physiological response in the cardiovascular system, including blood pressure, of a subject comprising administering to said subject the compound of formula (I-VII).

In other preferred embodiments, a compound of formula (selected from I thru VII) may have one or more pseudopeptide bonds. The abbreviation psi ("ψ") is the designation for a pseudopeptide bond (e.g., ("ψ")("CH₂NH")) indicates that the
10 amide ("C(O)NH-") bond of a peptide has been replaced by the reduced form ("CH₂NH-") or less preferably that of C(O)NH is replaced with any of CH₂-S, CH₂-O, CH₂-CH₂, CH₂-CO, or CH₂ CH₂). For example, Trp- ("ψ")(CH₂NH)Lys represents a tryptophan residue bonded to a lysine residue wherein the amide bond is reduced. A detailed discussion of the chemistry of pseudopeptide bonds is given in Coy *et al.*
15 (1988) *Tetrahedron* 44: 835-841, incorporated herein in its entirety by reference.

In other preferred embodiments, a therapeutically effective amount of a compound of formula (selected from I thru VII) and a pharmaceutically acceptable carrier substance, e.g., magnesium carbonate or lactose, together form a therapeutic composition capable of suppressing an NPY mediated physiological response. This
20 composition can be in the form a pill, tablet, capsule, liquid, or sustained released tablet for oral administration; or a liquid for nasal administration as drops or spray; or a liquid for intravenous, subcutaneous, parenteral, or intraperitoneal administration.

Another preferred form for administration biodegradable sustained-release composition for intramuscular administration to a subject in need of the composition. Preferably, the composition includes a lipophilic salt and is suitable for administration in the form of an oil emulsion or dispersion to a subject in need of the composition.

5 In yet another aspect, the invention features methods for controlling an NPY mediated physiological response in a subject; such methods involve administering one or more of the above mentioned compounds to a subject in a dosage effective to control blood pressure, the appetite, the libido, cardiovascular

10 function, and circadian rhythm.

The symbol A1, A2, A3, and the like; and Tyr, Lys or the like, as found in a peptide sequence herein stands for an amino acid residue, e.g., $=N-CH(R)-CO-$ when it is at the N-terminus, or $-NH-CH(R)-CO-$ when it is at any other position, where R denotes the side chain (or identifying group) of an amino acid or its residue.

15 For example, R is CH_2COOH for Asp, R is $-H$ for Gly, R is $-CH_2OH$ for Ser, R is $-CH_3$ for Ala and R is $-CH_2CH_2CH_2CH_2NH_2$ for Lys.

As set forth above and for convenience in describing this invention, the conventional and nonconventional abbreviations for the various amino acids are used. They are familiar to those skilled in the art; but for clarity are listed below. All

20 peptide sequences mentioned herein are written according to the usual convention whereby the N terminal amino acid is on the left and the C-terminal amino acid is on the right. A short line between two amino acid residues indicates a peptide bond.

Abbreviations (common):

Asp = D = Aspartic Acid
 Ala = A = Alanine
 Arg = R = Arginine
 Asn = N = Asparagine
 5 Cys = C = Cysteine
 Gly = G = Glycine
 Glu = E = Glutamic Acid
 Gln = Q = Glutamine
 His = H = Histidine
 10 Ile = I = Isoleucine
 Leu = L = Leucine
 Lys = K = Lysine
 Met = M = Methionine
 Phe = F = Phenylalanine
 15 Pro = P = Proline
 Ser = S = Serine
 Thr = T = Threonine
 Trp = W = Tryptophan
 Tyr = Y = Tyrosine
 20 Val = V = Valine

Abbreviations (uncommon):

Aoc = (8-aminooctanoic acid:
 Orn = Ornithine
 25 Nal = 2-naphthylalanine
 Thi = 2-thienylalanine
 Pcp = 4-chlorophenylalanine
 Bth = 3-benzothienylalanine
 Bip = 4,4'-biphenylalanine
 30 Tic = tetrahydroisoquinoline-3-carboxylic acid
 Aib = aminoisobutyric acid
 Anb = alpha-aminonormalbutyric acid
 Dip = 2,2-diphenylalanine
 Ac₆c = 1-aminocyclohexanecarboxylic acid
 35 D-Pal = beta-(3-pyridyl)alanine;
 Tcc = tetrahydrocarbolene-carboxylic acid
 Abu = alpha-aminonormalbutyric acid
 hArg(Pr)₂ = N,N'-guanidino-(dipropyl)-homoarginine
 Tic-OH = 1,2,3,4 tetrahydroisoquinoline-7-hydroxy-3-carboxylic acid
 40 Dip = 3,3-diphenylalanine
 2-Nal = 3(2-naphthylalanine)
 Tfp = Paratrifluoromethyl phenylalanine
 Fla = 3-(9-Fluorenyl)alanine
 Flg = 9-Fluorenylglycine
 45 Cit = Citrulline
 Adp = 2,5-diaminoadipic acid

Pim = 2,6-diaminopimelic acid
Sub = 2,7-diaminosuberic acid

5 The compounds of the invention are useful in treating any number of illnesses that involve eating disorders, cardiovascular function, alterations in sexual function, as well as disorders of sleep and circadian rhythms (see, e.g., *Harrison's Principles of Internal Medicine*, McGraw-Hill Inc., New York, 12th ed.).

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

10 The present invention is also directed to pharmaceutical compositions comprising a therapeutically-effective amount of a compound of formula (I-VII) and a pharmaceutically-acceptable carrier or diluent, as well as to a method of treating gastrointestinal disorders (including gallbladder disorders), central nervous system disorders, insulin-related disorders and pain, or of regulating appetite in humans and
15 lower mammals, by administration of a compound of formula (I-VII).

The terms "C2-C4-alkenyl" and "C2-C6-alkenyl" as used herein refer to a 2 to 4 to 6 straight- or branched-chain of carbon atoms which contains a carbon-carbon double bond, such as allyl, propenyl, butanol, isoprenyl and the like.

20 The terms "C1-C18-alkyl" as used herein refer to straight or branched chain alkyl radicals having from 1 to 18 carbon atoms including, but not limited to, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, pentyl, neopentyl hexyl, and the like.

The term "C6-C18-aryl" as used herein refers to phenyl or to a "bicyclic carbocyclic" group or "bicyclic carbocycle" having two fused carbocyclic
25 rings, each ring having 5, 6 or 7 carbon atoms, and each ring being fully saturated,

partially saturated or aromatic. Bicyclic carbocyclic groups include, but are not limited to, naphthyl, tetrahydronaphthyl, decalin, indanyl, indenyl and the like.

The term "C7-C18-arylalkyl" as used herein refers to an aryl group appended to a C1-C4-alkyl radical including, but not limited to, benzyl, phenethyl, naphthylmethyl and the like.

The term "bicyclic heterocycle" as used herein refers to a group having two fused rings, one or both of which are heterocyclic rings as defined herein. When both rings are not heterocyclic, the other ring is carbocyclic and is saturated, partially saturated or aromatic, preferably a benzene ring. Bicyclic heterocyclic groups can be unsubstituted or monosubstituted or disubstituted with substituents independently selected from hydroxy, halo, oxo (=O), amino, C1-C4-alkylamino, di-(C1-C4)-alkylamino, C1-C4-alkoxy, thio-C1-C4-alkoxy, carboxy, C1-C4-alkoxycarbonyl, C1-C4-alkyl, C3-C8-cycloalkyl, -OSO₃H and halo-C1-C4-alkyl. Examples of bicyclic heterocycles include indole, 5-hydroxyindole, quinoline, isoquinoline, tetrahydroisoquinoline, quinoxaline, benzimidazole, benzofuran, and the like.

The term "cyclo-C3-C10-alkyl" as used herein refers to an aliphatic monocyclic of 3 to 10 or bicyclic group having 6 to 10 carbon atoms including, but not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclooctyl, adamantyl, and the like.

The term "halo" or "halogen" as used herein refers to chloro, bromo, iodo or fluoro.

The term "halo-C1-C4-alkyl" as used herein refers to a lower alkyl radical in which one to three hydrogen atoms have been replaced by a halogen including, but not limited to, chloromethyl, 2-fluoroethyl, trifluoromethyl and the like.

- The term "monocyclic heterocyclic group" or "monocyclic heterocycle" as used herein refers to any 3- or 4-membered ring containing a heteroatom selected from oxygen, nitrogen and sulfur, or a 5- or 6-membered ring containing carbon atoms and one, two or three nitrogen atoms; one nitrogen and one sulfur atom; or one nitrogen and one oxygen atom; wherein the 5-membered ring has 0-2 double bonds and the 6-membered ring has 0-3 double bonds; wherein the
- 5 heterocycle" as used herein refers to any 3- or 4-membered ring containing a heteroatom selected from oxygen, nitrogen and sulfur, or a 5- or 6-membered ring containing carbon atoms and one, two or three nitrogen atoms; one nitrogen and one sulfur atom; or one nitrogen and one oxygen atom; wherein the 5-membered ring has 0-2 double bonds and the 6-membered ring has 0-3 double bonds; wherein the
- 10 nitrogen and sulfur heteroatoms may optionally be oxidized; and wherein the nitrogen heteroatom may optionally be quaternized. Heterocycles include, but are not limited to, pyridyl, imidazolyl, furyl, thienyl, pyrazinyl, pyrrolyl, pyrimidyl and the like. Heterocyclics may be unsubstituted or mono- or disubstituted with substituents independently selected from hydroxy, halo, oxo (=O), amino, C1-C4-alkylamino,
- 15 (C1-C4)2-alkylamino, C1-C4-alkoxy, thio-C1-C4-alkoxy, carboxy, C1-C4-alkoxycarbonyl, C1-C4-alkyl, C3-C8-cycloalkyl, -OSO₃H and halo-C1-C4-alkyl.

- The compounds of formula (I) contain two or more asymmetric carbon atoms and thus can exist as pure diastereomers, mixtures of diastereomers, diastereomeric racemates or mixtures of diastereomeric racemates. The present
- 20 invention includes within its scope all of the isomeric forms. In keeping with standard peptide nomenclature, J. Biol. Chem., 1969, 243:3557-59, abbreviations for amino acid residues are used herein.

It is noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

5 Administration

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated, the particular treatment and the particular mode of administration.

- It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy.

However, generally speaking the following guidelines will suffice.

- 15 When a compound of formula (I) is used as an agonist of NPY in a human subject, the total daily dose administered in single or divided doses may be in amounts, for example, from 0.001 to 1000 mg a day and more usually 1 to 1000 mg. Dosage unit compositions may contain such amounts of submultiples thereof to make up the daily dose.

- 20 The compounds useful in the present inventive method may be administered by any suitable means. One skilled in the art will appreciate that many suitable methods of administering the compounds to an animal in the context of the present invention, in particular a human, are available, and, although more than one

route may be used to administer a particular compounds, a particular route of administration may provide a more immediate and more effective reaction than another route.

- The composition according to the present invention may be formulated
- 5 for administration by any suitable route such as the oral, rectal, nasal, topical (dermal) or parenteral administration route. Thus, the composition may be in the form of tablets, capsules, suspensions, emulsions, solutions, injectables, suppositories, sprays, aerosols and in other suitable form.

- Formulations for oral use include tablets which contain the active
- 10 ingredient in admixture with non-toxic pharmaceutically acceptable excipients. These excipients may be, for example, inert diluents, such as calcium carbonate, sodium chloride, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, potato starch or alginic acid; binding agents, for example, starch, gelatin or acacia; and lubricating agents, for example, magnesium
- 15 stearate, stearic acid or talc. Other pharmaceutically acceptable excipients can be colorants, flavoring agents, plasticizers, humectants *etc.* The tablets may be uncoated or they may be coated by known techniques, optionally to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or
- 20 glyceryl distearate may be employed.

Formulations for oral use may also be presented as chewing tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft

gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil.

- Powders, dispersible powders or granules suitable for preparation of an aqueous suspension by addition of water are also convenient dosage forms of the
- 5 present invention. Formulation as a suspension provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents are, for example, naturally-occurring phosphatides, as *e.g.* lecithin, or condensation products of ethylene oxide with *e.g.* a fatty acid, a long chain aliphatic alcohol or a partial ester derived from
- 10 fatty acids and a hexitol or a hexitol anhydrides, for example, polyoxyethylene stearate, polyoxyethylene sorbitol monooleate, polyoxyethylene sorbitan monooleate *etc.* Suitable suspending agents are, for example, sodium carboxymethylcellulose, methylcellulose, sodium alginate *etc.*

- The present agents can also be administered in the form of liposomes.
- 15 As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to the tetrapeptide of
- 20 the present invention, stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic.

Methods to form liposomes are known in the art. See, for example, Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N.Y. (1976), p. 33 et seq.

- The pharmaceutical formulation may also be administered parenterally
- 5 (intravenous, intramuscular, subcutaneous or the like) in dosage forms or formulations containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions is well-known to those skilled in the art of pharmaceutical formulation.

- For parenteral use, the pharmaceutical compositions according to the
- 10 invention may comprise the thermogenic compounds in the form of a sterile injection. To prepare such a composition, the compounds are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-
- 15 butanediol, Ringer's solution and isotonic sodium chloride solution. The aqueous formulation may also contain one or more preservatives, for example, methyl, ethyl or n-propyl p-hydroxybenzoate.

- For the rectal application, suitable dosage forms for a composition according to the present invention include suppositories (emulsion or suspension
- 20 type), and rectal gelatin capsules (solutions or suspensions). In a typical suppository formulation, the compounds are combined with an appropriate pharmaceutically acceptable suppository base such as cocoa butter, esterified fatty acids, glycerinated gelatin, and various water-soluble or dispersible bases like polyethylene glycols and

polyoxyethylene sorbitan fatty acid esters. Various additives like *e.g.* enhancers or surfactants may be incorporated.

For the nasal application typical dosage forms for a composition according to the present invention include nasal sprays and aerosols for inhalation. In
5 a typically nasal formulation, the active ingredients are dissolved or dispersed in a suitable vehicle. The pharmaceutically acceptable vehicles and excipients and optionally other pharmaceutically acceptable materials present in the composition such as diluents, enhances, flavoring agents, preservatives, *etc.*, are all selected in accordance with conventional pharmaceutical practice in a manner understood by the
10 persons skilled in the art of formulating pharmaceuticals.

The pharmaceutical compositions according to the invention may also be administered topically on the skin for percutaneous absorption in dosage forms or formulations containing conventionally non-toxic pharmaceutically acceptable carriers and excipients including microspheres and liposomes. The formulations
15 include creams, ointments, lotions, liniments, gels, hydrogels, solutions, suspensions, pastes, plasters and other kinds of transdermal drug delivery systems. The pharmaceutically acceptable carriers or excipients may include emulsifying agents, antioxidants, buffering agents, preservatives, humectants, penetration enhancers, chelating agents, gelforming agents, ointment bases, perfumes and skin protective
20 agents.

Examples of emulsifying agents are naturally occurring gums, *e.g.*, gum acacia or gum tragacanth, naturally occurring phosphatides, *e.g.*, soybean lecithin and sorbitan monooleate derivatives.

Examples of antioxidants are butylated hydroxy anisole (BHA), ascorbic acid and derivatives thereof, tocopherol and derivatives thereof and cysteine.

Examples of preservatives are parabens and benzalkonium chloride.

Examples of humectants are glycerin, propylene glycol, sorbitol and

5 urea.

Examples of penetration enhancers are propylene glycol, DMSO, triethanolamine, N,N-dimethylacetamide, N,N-dimethylformamide, 2-pyrrolidone and derivatives thereof, tetrahydrofurfuryl alcohol and AZONE®.

Examples of chelating agents are sodium EDTA, citric acid and

10 phosphoric acid.

Examples of gel forming agents are Carbopol, cellulose derivatives, bentonit, alginates, gelatin and PVP.

Examples of ointment bases are beeswax, paraffin, cetyl palmitate, vegetable oil, sorbitan esters of fatty acids (Span), polyethyleneglycols, and

15 condensation products between sorbitan esters of fatty acids and ethylene oxide, *e.g.*, polyoxyethylene sorbitan monooleate (Tween).

The formulation and preparation of the above-mentioned compositions is well-known to those skilled in the art of pharmaceutical formulation. Specific formulation can be found in "Remington's Pharmaceutical Sciences" incorporated

20 herein by reference.

In one aspect the present invention relates to a method for treatment of overweight or obesity in individuals, in particular in humans or for reducing the

adipose tissue mass/lean mass body mass ratio of an individual, in particular a human or a domestic animal.

In the present context the term "overweight" is used as an indication of a body with a weight exceeding the "desirable weight", whereas the term "obesity" is used when the body weight is 20% or more above the "desirable weight". Desirable weights for humans are given by the Council on Scientific Affairs (1) defining the desirable weights for humans according to Metropolitan Height and Weight Tables as the midpoint of the range of the medium-frame individuals.

In another aspect, the present invention relates to a method for the treatment of diseases which are complications to overweight or obesity. These diseases or conditions include diabetes mellitus type II, hypercholesterolemia, hypertriglyceridaemia and hypertension.

In another aspect, the present invention also relates to a method of reducing adipose tissue mass/lean body mass ratio or treating overweight or obesity or complications thereof by means of subjecting the individuals to a diet regimen. The diet regimen into which the individuals may be subjected in connection with the administration of the composition may include a low carbohydrate, a low fat and a low energy regimen, *e.g.*, a diet of from 800-2500 kcal/day.

20 Veterinary Use

The compositions according to the invention can also be administered to domestic animals in order to improve the performance of the animal (daily weight gain and feed utilization) or to improve carcass quality or both. Carcass quality is

generally improved when the fat tissue mass/lean mass body mass ratio is decreased, *i.e.*, when the body content of meat is increased *e.g.*, at the expense of the body content of fat.

- The improvements in performance and carcass quality are suggested to
- 5 be caused by a reduced fat accretion and/or by an increased skeletal muscle accretion.
- In growing animals, the amount of lipid present is suggested to be governed by the relative rates of lipolysis and lipogenesis. Stimulation of lipolysis and/or inhibition of lipogenesis in fat tissue may lead to a reduced fat accretion. In vivo and in vitro studies with both pigs and ruminants may indicate that certain beta agonists stimulate
- 10 lipolysis and inhibit lipogenesis in fat tissue leading to a reduced fat accretion.

Administration to an animal of the compositions according to the present invention may be useful in order to increase the lean body mass at the expense of body fat, particularly in domestic animals like pigs, hogs, cattle, sheep and poultry.

- The composition may be given in admixture with the feed in a suitable dose
- 15 corresponding to the size of the animal.

Peptide Synthesis

- The peptides can be synthesized by any suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment
- 20 condensation or by classical solution addition. A detailed description of these methods is contained in "The Peptides, Vol. 1", Gross and Meinchenhofer, Eds., Academic Press, New York, 1979. Coupling methods employed include the carbodiimide method (1,3-dicyclohexylcarbodiimide [DCC], 1-(3-

- dimethylaminopropyl-3-ethylcarbodiimide hydrochloride [EDCI]) with the option of racemization preventing additives (1-hydroxybenzotriazole [HOBT]), the mixed anhydride method, the azide method, the acid chloride method, the symmetrical anhydride method, the use of bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl),
- 5 and the active ester method (N-hydroxysuccinimide esters, 4-nitrophenol esters, 2,4,5-trichlorophenol esters, and the like).

For example, the techniques of exclusively solid-phase synthesis are set forth in the textbook "Solid-Phase Peptide Synthesis" Stewart & Young, Freeman & Co., San Francisco, 1969, and are exemplified by the disclosure of U.S. Pat. No. 4,105,603, issued Aug. 8, 1978 to Vale *et al.* The fragment condensation method of

10 synthesis is exemplified in U.S. Pat. No. 3,972,859 (Aug. 3, 1976). Other available syntheses are exemplified by U.S. Pat. No. 3,842,067 (Oct. 15, 1974) and U.S. Pat. No. 3,862,925 (Jan. 28, 1975). All of the above references are incorporated herein by reference.

- 15 Synthesis by the use of recombinant DNA techniques, for purposes of this application, should be understood to include the suitable employment of a structural gene coding for all of the tripeptide to transform a microorganism, using an expression vector including a promoter and operator together with such structural gene, and causing such transformed microorganism to express the peptide or such a
- 20 synthetic peptide fragment. A non-human animal may also be used to produce the peptide by gene-farming using such a structural gene in the microinjection of embryos as described in U.S. Pat. No. 4,870,009 issued Sep. 26, 1989, incorporated herein by reference.

When the peptides are not prepared using recombinant DNA technology, they are preferably prepared using solid phase synthesis, such as that described by Merrifield, J. Am. Chem. Soc., 85, p 2149 (1964), although other equivalent chemical syntheses known in the art can also be used as previously

- 5 mentioned. Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected alpha-amino acid to a suitable resin as generally set forth in U.S. Pat. No. 4,244,946 issued Jan. 21, 1981 to Rivier *et al.*, the disclosure of which is incorporated herein by reference. Such a starting material for NPY analogs can, for example, be prepared by attaching alpha-amino- and side-chain-protected Tyr to a

10 BHA resin.

The compounds of the invention may be prepared by stepwise coupling of the amino acids or by coupling together fragments of dipeptide length or greater.

- Thus, the free carboxylic acid moiety from one amino acid or peptide fragment is activated and allowed to condense with the free nitrogen group of the second amino
- 15 acid or peptide fragment. The coupling reactions are conducted in solvents such as methylene chloride (CH_2Cl_2), tetrahydrofuran (THF), dimethylformamide (DMF) or other such solvents.

- During the coupling process, the non-participating carboxylic acids or amines on the reacting set of amino acids or peptide fragments are protected by a
- 20 protecting group which can be selectively removed at a later time if desired. A detailed description of these groups and their selection and chemistry is contained in "The Peptides, Vol. 3", Gross and Meinenhofer, Eds., Academic Press, New York, 1981, incorporated herein in its entirety by reference. Thus, useful protective groups

for the amino group are benzyloxycarbonyl (Cbz), t-butyloxycarbonyl (t-BOC), 2,2,2-trichloroethoxycarbonyl (Troc), t-amylloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-(trichlorosilyl)ethoxycarbonyl, 9-fluorenylmethoxycarbonyl (Fmoc), phthaloyl, acetyl (Ac), formyl, trifluoroacetyl, and the like.

- 5 Examples of useful protective groups for the carboxylic acid include esters, such as methyl, ethyl, benzyl, t-butyl, 2,2,2-trichloroethyl, allyl, 4-nitrobenzyl, and the like. Removal of these protecting groups may be accomplished selectively by employing various acid or base catalyzed hydrolytic, hydrogenolytic, thermal or dissolving metal conditions.

- 10 Generally, peptides will be synthesized by stepwise solid phase methodology developed by using an automated Applied Biosystem Model 430A peptide synthesizer. Tertiary butyloxy-carbonyl (Boc) amino acids with benzyl or halobenzyl based side chain protecting groups (Asp & Glu with OBzl; Ser & Thr with Bzl; Cys with pMeBzl; Tyr with 2BrZ; Lys with 2ClZ; Arg with Tos; His with Bom;
- 15 Trp with CHO) will be used in conjunction with phenylacetamidomethyl (PAM) resin. In the case of the synthesis of peptide amides, benzyldrylamine (BHA) or paramethylbenzylhydrlamine (MBHA) will be used instead of PAM resin.

- Boc-aminoacid-PAM-resin, using Boc-aminoacyloxy-methyl-phenylacetic acid and aminomethyl resin, is available commercially. The Boc-
- 20 aminoacid-PAM-resin thus prepared eliminates the possibility of chain termination by tri-fluoroacetylation. Attachment to BHA or MBHA resin will be performed by way of preformed symmetrical anhydride.

Coupling and deprotection functions are generally carried out automatically by the instrument. The standard program provided by the

manufacturers are modified to incorporate a double coupling procedure, first in DMF and then in CH_2Cl_2 . Altering the polarity of the solvents improves the coupling. All

- 5 amino acids, except Asn, Gln and Arg, will generally be coupled as preformed symmetrical anhydrides. Asn, Gln and Arg are double coupled as preformed 1-hydroxy-benzotriazole esters to avoid side reactions. Resin samples taken during these reactions may be assayed by quantitative procedure to determine the degree of coupling. Other standard cleaving reagents and conditions for removal of specific
- 10 alpha-amino protecting groups may be used as described in Schroder & Lubke, "The Peptides" Vol. 1, pp 72-75 (Academic Press 1965), incorporated herein in its entirety by reference.

In the case of coupling unusual amino acids, suitable conditions (solubility, coupling times) will be first developed before using in automated mode. In

- 15 some cases these couplings will be carried out manually (eg: pseudopeptides, N-Me-amino acids). Pseudopeptide bonds will be incorporated by the methods described eralier. The t-Boc-amino acid aldehyde will be obtained by reducing N-methoxy-N-methylamide derivatives of Boc-amino acids with LiAlH_4 . The aldehyde obtained will be reacted immediately with the α -amino group of the peptide attached to the
- 20 resin in DMF containing 1.0% HOAC in the presence of an equivalent amount of NaBH_3CN . At the end of the reaction, the presence of secondary amine is tested for with ninhydrin (wine-red color). The secondary amine formed will then be blocked by reacting with 2 equivalents of Z(2-Cl)OSU , 2 equivalents of HOBT, and 4

equivalents of diisopropylethylamine until ninhydrin gives a yellow color. This way the formation of branched peptide is prevented. Coupling of sterically hindered amino acids (eg: N-Me-amino acids, CaMeLeu, Aib) will be effected by a HOAT or HATU which has been shown to be superior to BOP/HOBT.

- 5 For the final cleavage, the N- α -Boc group and the Nin-CHO will be first removed with 50% TFA/CH₂Cl₂ and 20% piperidine-DMF from the protected peptide resin before detaching the target peptide using HF containing p. cresol (5%). If Cys and Met are present, p. thicresol (2.5%) will also be added to the HF reaction mixture. If problems are encountered during the standard HF method, then the
- 10 "low/high" HF procedure will be used.

- The materials are then purified. After initial fractionation on Sephadex G-25, the peptide material will be subjected to reversed phase high performance liquid chromatography (RPLC) on C₁₈ Vydac columns. However, peptides may be first subjected to ion exchange chromatography before RPLC, depending upon the
- 15 heterogeneity of the crude peptide. The homogeneity of the purified product may be confirmed by analytical RPLC using two different solvent systems, amino acid anaynsis, complete sequencing, and mass spectral analysis.

- For analysis, the peptide resins are hydrolyzed using 12N HCl/HOAc/phenol (2:1:1) for 24 hours at 110°C. The free peptides are hydrolyzed
- 20 for 24 hours in 6N HCl containing 0.1% phenol or 4N methane sulfonic acid at 110°C and are quantified on a Waters Pico Tag system. Peptide hormones and fragments are then subjected to complete sequencing on an automated gas phase sequencer (Applied Biosystem, Model 470A).

- For the production of a compound of the invention where any one or several of the constituent amino acids bear an N-alkyl group, specifically methyl, the corresponding N-alkyl amino acid can be prepared via the method described by Benoiton (*Can. J. Chem.*, 1977, 55:906) or Shuman ("Peptides: Proceedings of the 7th American Peptide Symposium", D. Rich, E. Gross, Eds., Pierce Chemical Co., Rockford, Ill. 1981, p 617), wherein the t-BOC- or Cbz-protected amino acid is treated with a base in the presence of a chelating agent such as a crown ether and then quenched with methyl iodide. An alternative method described by Freidinger (*J. Org. Chem.*, 1983, 48:77), in which triethylsilane reduction of the oxazolidinone of an amino acid directly produces the N-methyl derivative may also be utilized.

- The reduced carbonyl amide bond surrogates can be prepared in a manner similar to that described by Martinez (*J. Med. Chem.* 1987, 30:1366). The N-alpha -t-BOC protected amino acid (with appropriate protection of side chain functional groups) is converted to the 3,5-dimethylpyrazolide, which is then reduced with lithium aluminum hydride. The resulting aldehyde is then allowed to condense with an amino acid or peptide bearing a free amino terminus. Reduction of the Schiff base which is formed as a result of the condensation is accomplished using sodium cyanoborohydride to yield the desired compound having a reduced amide bond.

- Functionalization of the epsilon -amino group of the lysine (Lys) or homologous (e.g., Orn) residue is achieved via activation of the acid fragment as the active ester (N-hydroxysuccinimide, 2,4,5-trichlorophenol, etc.) or, if no other free carboxylic acid function is present on the peptide, coupling using any of the methods mentioned above is applicable. In addition, the functionalization of the epsilon -

amino group may be accomplished by reaction with various alkyl and aryl isocyanates, as well as alkyl and aryl isothiocyanates.

- The sulfuric acid esterification of the phenolic residues may be conducted using a variety of known reagents such as the pyridine-sulfuric anhydride
- 5 or the pyridine-sulfur trioxide complex. Use of pyridinium acetyl sulfate as described by Penke and Rivier ("Proceedings of the 8th American Peptide Symposium", V. Hruby, D. Rich, Eds., Pierce Chemical Company, Rockford, IL; 1983; p. 119), may also be applied to prepare the sulfuric acid ester derivative of the tetrapeptides.

- The compounds of the present invention can be used in the form of
- 10 salts derived from inorganic or organic acids. These salts include but are not limited to the following: acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, cyclopentanepropionate, dodecylsulfate, ethanesulfonate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, fumarate, hydrochloride,
- 15 hydrobromide, hydriodide, 2-hydroxy-ethanesulfonate, lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate. Also, the basic nitrogen-containing groups can be quaternized with such agents as loweralkyl halides, such as methyl, ethyl,
- 20 propyl, and butyl chloride, bromides, and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl, and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides like benzyl and phenethyl

bromides, and others. Water or oil-soluble or dispersible products are thereby obtained.

The pharmaceutically acceptable salts of the present invention can be synthesized which contain a basic or acidic moiety by conventional chemical methods. Generally, the salts are prepared by reacting the free base or acid with stoichiometric amounts or with an excess of the desired salt forming inorganic or organic acid or base in a suitable solvent or various combinations of solvents.

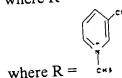
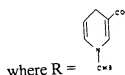
Examples of preferred salts are those with therapeutically acceptable organic acids, *e.g.*, acetic, lactic, maleic, citric, malic, ascorbic, succinic, benzoic, or pamoic acid, as well as polymeric acids and salts with inorganic acids such as the hydrohalic acids, *e.g.*, hydrochloric and sulfuric acids.

In addition, pseudopeptide bonds may, if desired, may be introduced at various positions, *e.g.*, between amino acid residues A1-A2 or between residues A2-A3. Optically pure Boc-AA-CHO can be obtained in good yields and coupled directly to the -NH₂ group of the peptide resin by published methods (Sasaki *et al.*, *Peptides* 8:119-121, 1987; Fehrentz *et al.*, *Synthesis* pp.676-678, 1983). The secondary amine in the pseudopeptide bond is capped with Z(2-Cl). This is introduced by reacting the peptide resin with Z(2-Cl)-OSU (2 equiv.), HOBT (2 equiv.) and DIEA (4 equiv.) for 10-60 min. The red wine color of ninhydrin with secondary amine turns yellow at the end of capping. Exemplary compounds of the present invention include:

1. Ac-Phe-Arg-Trp-NH₂
2. Ac-Trp-Arg-Phe-NH₂
- 25 3. Ac-Trp-Arg-Tyr-NH₂
4. Ac-Trp-Ctr-Tyr-NH₂
5. Ac-Trp-(Ψ)-Arg-Tyr-NH₂

6. Ac-Trp-Arg-Tyr-(Ψ)-NH₂
7. Ac-Arg-Tyr-NH₂
8. Ac-Trp-Arg-Tyr-[O(PO(OH)₂)]-NH₂
9. (HO)₂PO-CH₂-CO-Trp-Arg-Tyr-NH₂
- 5 10. Ac-Arg-His-Tyr-NH₂

11. R-CO-Trp-Arg-Tyr-NH₂



12. R-CO-Trp-Arg-Tyr-NH₂
13. Ac-Trp-Arg-D-Tyr-NH₂
14. Ac-Trp-D-Arg-Tyr-NH₂
- 10 15. Ac-D-Trp-Arg-Tyr-NH₂
16. Ac-D-Trp-D-Arg-D-Tyr-NH₂
17. Trp-Arg-Tyr-NH₂
18. Ac-Trp-Arg-Tyr-OH
19. Ac-Trp-Arg-Tyr-NH-Me
- 15 20. Ac-Trp-Arg-Tyr-NH₂
21. Ac-Arg-Tyr-NH₂
22. Bis(34/34') {[Cys34]NPY(34-36)}
23. Ac-Gln-Arg-Tyr-NH₂
24. H-Gln-Arg-Tyr-NH₂
- 20 25. Ac-Trp-h.Arg((Pr)₂-Tyr-NH₂
26. [Des-α-Amino-Trp]-Arg-Tyr-NH₂
27. Ac-Trp-Arg-Tic(OH)-NH₂
28. N-α-Indole-3-acetyl-Arg-Tyr-NH₂
29. Ac-Tcc-Arg-Tyr-NH₂
- 25 30. Ac-Pro-Arg-Tyr-NH₂
31. Ac-Nal-Arg-Tyr-NH₂
32. Ac-Bth-Arg-Tyr-NH₂
33. Ac-Trp-Arg-Tyr-NH-CH₃
34. Ac-D-Trp-Arg-Tyr-NH₂
- 30 35. Ac-Trp-D-Arg-Tyr-NH₂
36. Ac-Trp-Arg-D-Tyr-NH₂
37. Ac-D-Trp-D-Arg-D-Tyr-NH₂
38. Ac-[-Trp-Arg-Tyr-]₂-NH₂
39. [Ac-Cys-Trp-Arg-Tyr-NH₂]₂ ✓
- 35 40. [-CH₂CO-Trp-Arg-Tyr-NH₂]₂
41. Dep-His-Trp-Arg-Glu-Arg-Tyr-NH₂
42. Leu-His-Trp-Arg-Glu-Arg-Tyr-NH₂
43. Cyclo[-Trp-Arg-Nva-Arg-Tyr-]
44. RCO-Trp-Arg-Tyr-NH₂
- 40 45. Ac-Trp[CH₂NH]Arg-Tyr-NH₂
46. Ac-Trp-Arg-[CH₂NH]Tyr-NH₂

2 ✓
3 ✓
4 ✓
5 ✓

47. Ac-Arg-Tyr-NH₂
48. Ac-Trp-Arg-NH₂
49. Ac-Tcc-Arg-Tic(OH)-NH₂
50. Cyclo[-Trp-Arg-Tyr-Trp-Arg-Tyr-]
51. N- α -Ac-Cyclo(29/34)[D-Cys 29,34,Trp 32]NPY(29-36)-NH₂
52. Cyclo(30/34)[Leu30,Trp32,Glu34,]NPY(30-36)-NH₂
53. Cyclo(30/34)[Dap30,Trp32,Glu34,]NPY(30-36)-NH₂

- Other analogs of the invention can be prepared as above and tested for
- 10 their biological activity effectiveness as antagonists or agonists using the methods described below and those commonly known in the art.

FUNCTIONAL ASSAYS

Animals. Cell Lines and Cultures. and Reagents

- 15 Any suitable *in vivo* or *in vitro* system may be utilized to assay and test the effectiveness of the compounds of the invention. Such assays may employ *in vivo* methods for evaluating physiological responses, *e.g.*, blood pressure, renovascular function, feeding behavior, or circadian rhythm, or *in vivo* biochemical systems evaluating receptor binding in a suitable cell line, *e.g.*, SK-N-MC (ATCC
- 20 HBT 10) or SK-N-BE(2) (Barnes *et al. In Vitro*, 17: 619 631, 1981); or in isolated cells, *e.g.*, cells isolated from the spleen, kidney, heart or brain. A number of *in vivo* and *in vitro* biochemical systems known to those skilled in the art are available for testing antagonists to hypothalamic NPY receptors, *e.g.* the Y-1, Y-2, and Y-3 receptor categories. Described below are assay methods which can be utilized with cell lines
- 25 such as SK-N-MC and SK-N-BE2 or isolated cardiac membranes which possess the high-affinity hypothalamic NPY receptor sites. Other systems are also known for evaluating NPY antagonists to the hypothalamic NPY receptor, *e.g.* VSM cells

- (Sheikh *et al.*, *Am. J. Physiol.* 260: G250-G257, 1991) and HEL cells (Motulsky *et al.*, *Amer. J. Physiol.* 255: E880-E885, 1988); Y-2 receptor, *e.g.*, kidney (Sheikh *et al.*, *Am. J. Physiol.* 26:F978-F984), spleen (Lunberg *et al.*, *Eur. J. Pharmacol.* 145:21-29, 1988), dorsal root ganglion (Bleakman *et al.*, *Br. J. Pharmacol.* 103:1781-1789, 1991)
- 5 and hippocampal cells (Sheikh *et al.*, *J. Biol. Chem.* 265:8304-8310, 1990); and Y-3 receptors, *e.g.*, in cardiac ventricular membranes (Balasubramaniam *et al.*, *Peptides* 11: 545-550, 1990), chromaffin cells, rat gastric mucosa (Michel, M.C., *Trends in Pharmacol. Sci.* 12: 389-394, 1991) and brain stem.

10 In Vitro Biochemical Assays

- The ability of the compounds of the invention to act as antagonists of NPY can be demonstrated by any number of methods known in the art. For example, the compounds can be shown to compete with iodinated neuropeptide Y for receptors using the methods described by Lundberg *et al.* (*Eur. J. Pharmacol.* 145: 21-29, 1988);
- 15 Gordon *et al.* (*J. Neurochemistry* 55:506-513, 1990); Walker *et al.* (*Mol. Pharmacol.* 34:779-792, 1988); Balasubramaniam *et al.* (*Peptides* 10:1283-1286, 1989).

- In one example demonstrating antagonists to hypothalamic NPY receptors, rat hypothalamus was isolated and the membranes were prepared for binding and adenylate cyclase studies according to standard methods (Unden *et al.* 1984. *Eur.*
- 20 *J. Biochem* 145: 525-530; Westlind-Danielsson *et al.*, *Neurosci. Lett.* 74: 237-242 (1987)). Displacement studies are performed in a total volume of 0.25 ml 20 mM HEPES buffer, pH 7.4, containing 1% bovine serum albumin, 0.1% bacitracin, 300 μ M PMSF and 5 KIU/ml aprotinin. In a standard assay, 100 μ g of membrane/tube is

incubated in a shaking water bath at 24_C for 45 min with [¹²⁵I-Tyr]-NPY (20,000 CPM) as described by Balasubramaniam *et al.*, (*Peptides* 11: 545-550, 1990), in the presence of increasing concentrations of NPY (10 μOsM). At the end of incubation, 1.0 ml of iced cold buffer is added, centrifuged at 10,000 x g for 10 min, and the supernatant removed by aspiration. The tube containing the pellet is counted for bound radioactivity in a micromedic gamma counter.

An example of assaying adenylate cyclase activity of hypothalamic and cerebral cortex membranes is now described.

Adenylate cyclase activity of the hypothalamic and cerebral cortex membranes is determined by incubating 50 μg of membranes in a total volume of 0.20 ml Tris-HCl 30 mM pH 7.4 buffer containing 150 mM NaCl, 8.25 mM MgCl₂, 0.75 mM EGTA, 1.5 theophylline, 20 μg/ml aprotinin, 100 μg/ml bacitracin, 1 mg/ml bovine serum albumin, 1 mM ATP, 20 mM creatine phosphate, 1 mg/ml phosphocreatine kinase, 10 μM isoprenalol, 10 μM GTP, and various concentrations of peptides (0-10 μM). After incubating the mixture at 35_C for 15 min in a shaking water bath, the reaction is arrested by the addition of 100 μM EDTA and boiling for 3 min. cAMP is extracted and quantitated by radioimmunoassay. All the points in the binding and adenylate cyclase are the means of at least three parallel experiments performed in duplicate.

In Vivo Assays

Any suitable *in vivo* model system can be used to evaluate the antagonistic properties of the compounds of the invention. Such models, without

limitation, include those used to evaluate feeding and memory behavior (Flood *et al.*, *Peptides* 10:963-966), and vasoconstriction and hypertension (Balasubramaniam *et al.* *Biochemm et Biophys Acta* 997: 176-188, (1989)).

- Thus, in one example, feeding studies are performed using Sprague
- 5 Dawley rats (350-450 g) with paraventricular hypothalamic cannulae to investigate effects of NPY analogs (Chance *et al.*, *Peptides* 10: 1283, 1286 (1989)).

- The following Examples set forth preferred methods for synthesizing tripeptides by the solid-phase technique and generally is in accordance with the procedure set forth in U.S. Pat. No. 4,415,558 to Vale, *et al.*, issued Nov. 15, 1983,
- 10 the disclosure of which is incorporated herein by reference.

EXAMPLES

Example I

- The synthesis of N-alpha-Ac-Trp-Arg-Tyr-NH₂, is conducted in a
- 15 stepwise manner on a methylbenzhydrylamine hydrochloride resin, such as available from Bachem, Inc., having a substitution range of about 0.1 to 0.5 mmoles/g resin. The synthesis is performed on an automatic Applied Biosystems, Model 430A, peptide synthesizer using the program supplied by the manufacturer. Briefly, MBHA resin is placed in the reaction vessel of the peptide synthesizer, and the amino acids,
- 20 Boc-Tyr(2BrZ)-OH, Boc-Arg(Tos)-OH and Boc-Trp(CHO)-OH, are sequentially coupled using preformed HOBt ester (2.0 mmol) according to the program provided by the ABI 430A synthesizer. At the end of the synthesis, alpha-amino group is acetylated with acetic anhydride and the in-CHO group removed by stirring with 20%

- 5 piperidine in DMF for 2 hours. The peptide is detached from the resin by treating with HF containing 5% p. cresol for about 1 h at about -2°C. HF is evacuated under vacuo and the residue transferred to a fritted funnel with diethyl ether and is washed repeatedly with diethyl ether. The crude peptide extracted with acetic acid (10 ml x 2) is diluted to 10% and lyophilized. The peptide is then purified to homogeneity by reversed phase chromatography on a C-18 semi-preparative column (250 x 10 mm) using the buffer systems 0.1% TFA-H₂O and 60% CH₃CN in 0.1% TFA-H₂O. The peptides are characterized by amino acid and mass spectral analysis.

10 Example II

- The peptide N-alpha-Ac-Trp-[CH₂NH]-Arg-Tyr-NH₂ is synthesized using the method as described in Example I. However, Boc-Trp-CHO, (*Synthesis* 676-679, 1983), is coupled to H-Arg(Tos)-Tyr(2br-Z)-MBHA-Resin by reductive alkylation in the presence of 1.0% HOAc and 1.0 equiv. of NaBH₃CN in DMF. The crude peptide obtained by HF cleavage is purified as described earlier. Boc-Arg(Z)2-OH is used to prepare the corresponding aldehyde and synthesize N-alpha-Ac-Trp-Arg-[CH₂NH]-Tyr-NH₂.

Example III

- 20 A peptide dimer is synthesized using the general method as described in Example I, except that after the synthesis of Trp(CHO)-Arg(Tos)-Tyr(2-Br-Z)-MBHA-Resin, the peptide resin is reacted with half an equivalent N, N-Bis-Boc-Cystine, succinic acid, maleic acid or N₂, N₆-di-Boc-Pim (Pim = 2,6-diaminopimelic

acid), in the presence of 1 equivalent of DCC, HOBT and DIEA until the ninhydrin test is negative. The uncoupled alpha-amino group may be acetylated with acetic anhydride. The peptides are then cleaved and purified as described above.

5 Example IV.

To study the feeding patterns of animals treated with the tripeptides, rats with intrahypothalamic cannulae are used. Sprague-Dawley rats weighing about 250-350 g are anesthetized with pentobarbital (45 mg/kg) and 24 ga stainless steel cannulae are surgically implanted into the perifornical hypothalamus at the following stereotaxic coordinates taken from the interaural line: A=6.5, L=0.8, V=-2.0 mm. Investigations are performed two weeks after surgery.

A total of 32 rats are divided into four groups in the initial screening. These include control (artificial cerebral spinal fluid, CSF), T-83 (Ac-Tcc-Arg-Tyr-NH₂), T-84 (N-a-indole-3-acetyl-Arg-Tyr-NH₂), and T-91 (Ac-Trp-Arg-Tic(OH)-NH₂). Artificial CSF (CSF, 0.5 µl) or peptides (0.5-20.0 µg) in CSF are injected via the cannulae directly into the hypothalamus and the food intake of rat chow is monitored for 1, 2 and 4 hour periods. Water is available throughout the experiment. The results are shown in Fig. 1.

20 Example V.

A feeding experiment is performed as in Example IV except that the 32 rats are divided into four groups which include control (artificial cerebral spinal fluid,

CSF), T-45 (Bis(34/34') {[Cys34]NPY(34-36)}), WX-66 (Ac-D-Trp-Arg-Tyr-NH₂), and WX-126 (Ac-Gln-Arg-Tyr-NH₂). The results are shown in Fig. 2.

Example VI.

5 A feeding experiment is performed as in Example IV except that 15 rats are divided into two groups which include control (artificial cerebral spinal fluid, CSF) and T-34 (N-Ac-Trp-Arg-Tyr-NH₂). Food intake of rat chow is monitored for 1,2,4 and 24 hour periods. The results are shown in Fig. 3.

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